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LEAKAGE OF PHOSPHATASES AND FERTILITY OF BUCK SEMEN CRYOPRESERVED UNDER DIFFERENT FREEZING MODES

S Sharma [1*], NK Sharma[2], N Kataria[3] and NK Sinha[4]

[1]Department of Veterinary Obstetrics & Gynecology, College of Veterinary and Animal Science, Bikaner, India
[2]Mobile Veterinary Surgery and Infertility Unit, Animal Husbandry Department, Government of Rajasthan, Bikaner, India
[3]Department of Veterinary Physiology, College of Veterinary and Animal Science, Bikaner, India
[4]Central Institute for Research on Goats (ICAR), Makhdoom, Farah, (Mathura), India

[*]Corresponding Author E-mail: dr.sunandasharma@yahoo.in

Abstract

Present study was conducted on 160 ejaculates collected at weekly interval by artificial vagina method from 13 adult Sirohi bucks. Pooled ejaculates were diluted with Tris-egg yolk-citric acid-fructose-glycerol extender (1:4), filled and sealed in French mini straws. Few straws of diluted semen were thawed (40°C/15 seconds) and assessed for acid and alkaline phosphatases (ACP and AKP) in seminal plasma of diluted semen (control group). Remaining semen straws were randomly grouped to constitute freezing mode groups (M_1, M_2, M_3, and M_4) and processed further for cryo-preservation of semen. Accordingly diluted semen straws were cooled @-4° C/minute from 25°C up to 5°C thereafter equilibrated at this temperature for 2 hours and then frozen up to -160° C @ 15, 20, 25 and 30° C/minute for M_1, M_2, M_3 and M_4 groups respectively. These frozen straws were held at this temperature for 2 minutes then stored separately in LN_2. After 7 days of storage, straws from each freezing mode group were thawed and assessed for ACP and AKP in seminal plasma. *In-vivo* fertility trials were also conducted with straws from control (fresh diluted semen) as well as freezing mode groups (frozen at different freezing rates). Least square analysis of variance for the data obtained revealed highly significant ($P \leq 0.01$) rise in the seminal plasma ACP and AKP enzyme levels in frozen thawed semen as compared to that in fresh diluted semen. The Values of ACP and AKP also differed significantly ($P \leq 0.05$) among all the freezing mode groups wherein lowest values of ACP were observed in M_3 group followed by M_4, M_2 and M_1 groups in increasing order whereas, lowest values of AKP were observed in M_3 followed by M_2, M_4 and M_1 groups in increasing order. Highest fertility rates were observed with semen from M_3 followed by M_2, M_4 and M_1 groups. On the basis of enzyme leakage and *in-vivo* fertility trials, the optimum freezing rate for cryopreservation protocol was arrived at 25°C/minute.

Keywords: Buck Semen, Freezing rates, Seminal Plasma phosphatases, ACP, AKP

Introduction

Sirohi goat is well recognized dual purpose breed having better performance for average daily gain as compared to Kutchi and Marwari goats; hence it could be employed as an improver breed for increasing meat and milk production in medium and small sized goats (Acharya, 1992 and Groot *et al.,* 1992). Genetic potential for production traits in goats could favorably be augmented by breeding strategies covering a large numbers of doe with germplasm of genetically superior bucks. Artificial insemination (A.I.) with frozen semen is a desirable tool for genetic improvement in animals (Nuti, 1997) but frozen semen is not utilized on a wide spread basis for A.I. in goats because available cryopreservation protocols do not provide an acceptable level of fertility (Parks and Graham, 1992). Success of artificial insemination programme requires a suitable deep-freezing methodology for cryopreservation of diluted male germplasm without or with least compromised fertilizing ability. Review of available literature reveals that freezing protocols for cryopreserving buck semen have extensively been studied for variables like semen extenders, dilution rates, equilibration period, pellet *versus* straw freezing, thawing temperatures, thawing rates and semen additives etc., but there is meager any report about effect of freezing rates on post thaw semen quality (Daskin *et al.*, 2011 Naing *et al.* 2011 and Nor-Ashikin, Abdullah, 2011, Ansari *et al.,* 2012 and Beltran *et.al.*, 2013). Phosphatases are found in seminal plasma, acrosomes, sub-acrosomal space, post nuclear cap, cytoplasmic droplets and tail specially the mid piece of spermatozoa (Guraya, 1987). Seminal plasma phosphatases are derived from the secretions of accessory sex glands (Abdou *et al*., 1974) and are involved in transport of calcium ions from external fluids of female genital tract (Restall and Wales, 1968).

The calcium ions in turn play critical and multifaceted role in the process of fertilization (Yanagimachi 1981, Metz and Manroy 1985 and Guraya 1987). Acrosomal phosphatases play important role in sperm capacitation and acrosomal reaction whereas, intracellular phosphatases play important role in cell metabolism (Guraya 1987). Semen freezing has been reported to alter cell membrane permeability and damage to acrosome causing leakage of intra-cellular enzymes (Zanveld *et al.* 1971 and McRorie and Williams 1974). Present study was therefore conducted to investigate suitable rate of freezing that is least detrimental to the spermatozoa with comparative low leakage of phosphatases into seminal plasma so that the freezing protocol for buck semen could further be improved.

Materials and Methods

One Hundred Sixty ejaculates from 13 adult Sirohi bucks were collected at weekly interval by A-V method. Pooled ejaculates were diluted @ 1:4 with tris-egg yolk-citric acid-fructose-glycerol extender at room temperature (25^{o}C) and filled in french mini straws. Few straws of diluted semen were thawed (40^{o}C/15 sec.) and used for assessing phosphatases (ACP and AKP) in seminal plasma from fresh diluted semen (control group) of sirohi bucks, whereas, remaining straws were further processed for cryopreservation under different freezing rates. Accordingly diluted semen straws were cooled from 25^{o}C to 5^{o}C under controlled rate of cooling (0.4^{o}C/minute) in a programmable biofreezer (Kryo-440-1.7;Model 10/1.7; Series III; Planer Product Ltd., Middlesex), thereafter equilibrated at 5^{o}C for 2 hours. These equilibrated straws were frozen from 5^{o}C to -160^{o}C under controlled rates of freezing (15, 20, 25 and 30^{o}C/minute for M_1, M_2, M_3 and M_4 group respectively). These frozen straws were then immerged and stored in LN_2 (-196^{o}C). Semen straws from each group were thawed at 40^{o}C for 15 seconds then centrifuged @ 3000 r.p.m. for 15 minutes, supernatant seminal plasma was collected thereafter alkaline phosphatase was estimated as per the method described by Kind and King (1954), whereas, acid phosphatase was assessed by King's Method (King and Jagatheesan, 1959) using the diagnostic reagent kits supplied by Span diagnostics, Ltd, Surat, India. A minimum of 10 observations were recorded for each enzyme from semen straws of each group. The results obtained were statistically analyzed for arriving at Mean $\pm$ S.E. values of enzymes for each group as well as coefficient of variation among different groups. The data obtained were subjected to mixed model least square and maximum likelihood computer programme PC-1 for studying the analysis of variance (Harvey, 1987). The mean value of enzymes among all the groups were compared as per Duncun's multiple range test (Snedecor and Cochran, 1980). Fertility trials were conducted in 53 doe wherein natural estrus was detected by parading an approned buck every morning and evening. Doe in natural heat was inseminated twice (at an interval of 8-10 hours) by deep cervical trans-vaginal insemination with diluted semen from control group as well as frozen thawed semen from each freezing mode group. A minimum of 10 doe were inseminated with semen from each group. The freezing rate with least compromised damage to sperms was arrived at on the basis of comparatively low enzyme leakage and high fertility rate.

Results and Discussion

The values of acid and alkaline phosphatases in fresh diluted as well as frozen thawed semen from different groups as well as mean $\pm$ S.E. and coefficient of variations for respective values have been depicted in Table-1. Least Squares analysis of variance showing effect of freezing rates on leakage of seminal Plasma phosphatases have been presented in Table-2 showing comparisons between the groups.

Fresh Diluted Semen

Acid and alkaline phosphatases (ACP and AKP) in fresh diluted semen from control group ranged from 22.5 to 35.625 and 45.8333 to 64.1667 with respective Mean $\pm$ S.E. (CV%) 28.4180 $\pm$ 0.9086 (12.7893) and 56.375 $\pm$ 2.166 (12.1499) KA unit per 0.9221 x 10^9 spermatozoa (Table-1). The ACP: AKP ratio observed in present study was 1:1.98.

In present study values of acid and alkaline phosphatases in seminal plasma of Sirohi bucks were in accordance with those reported by Varshney et al. (1978), Kapila (1992) and Kale and Tomer (2000). The ratio of AKP: ACP observed in present study (1.98:1) was in close approximation to that reported by Kale and Tomer (2000), whereas higher ratios have been reported in semen of barbari (Varshney *et al.* 1978) and Jamunapari (Kapila, 1992) breeds of bucks.

The observed values of acid phosphatase in seminal plasma of sirohi bucks were lower than those reported in diluted semen of Barbari bucks (Sinha et al., 1999-2000 and Tiwari 2000). Alkaline phosphatase contents of seminal plasma observed in present study were lower than those reported in barbari bucks (Varshney et al., 1978) and black-bengal x beetal bucks (Patil and Raja 1978). Mean $\pm$ S.E. values of AKP in present study were higher than those reported in black-bengal x beetal bucks by Singh et al. (1996).

These variations in ACP and AKP contents of seminal plasma could be due to differences in breeds (Sinha et al., 1985; Sinha et al., 1988 and Sinha et al., 1999-

2000), age of bucks (Tiwari, 2000), seasons of semen collection (Baruah et al., 1992 and Kale and Tomer, 2000), sperm concentration (Kakar and Anand, 1984); rates of dilution and composition of diluents, (Singh et al., 1993). Individual variations between the bucks of same breed have also been reported by Tuli et al . (1991) and Kale and Tomer (2000).

Table-1: Phosphatases in fresh diluted semen (control group) and frozen thawed semen (freezing mode groups)

S. N.	Phosphatases	GroupWise Seminal Plasma Enzyme (KA / 0.9221 x 10^9 spermatozoa)				
		M_1 (15^0 C/mt)	**M_2 (15^0 C/mt)**	**M_3 (15^0 C/mt)**	**M_4 (15^0 C/mt)**	**Control (15^0 C/mt)**
1	Acid Phosphatase	56.2500	42.1875	37.5000	42.1875	29.0625
		55.3125	43.1250	37.5000	44.0625	22.5000
		57.1875	44.0625	37.5000	45.0000	24.3750
		59.0625	45.0000	38.4375	46.8750	35.6250
		60.0000	43.1250	40.3125	41.2500	31.8750
		60.9375	44.0625	40.3125	43.1250	26.2500
		56.2500	45.0000	40.3125	44.0625	30.0000
		54.3750	45.9375	41.2500	45.9375	28.1250
		55.3125	46.8750	38.4375	40.3125	23.4375
		58.1250	47.8125	38.4375	42.1875	33.7500
		59.0625	46.8750	38.4375	43.1250	27.1875
		60.0000	47.8125	39.3750	45.0000	30.000
		56.2500	41.2500	41.2500	39.3750	25.3125
		57.1875	48.7500	41.2500	41.2500	29.0625
		58.1250	44.0625	41.2500	42.1875	30.9375
		60.9375	45.9375	42.1875	44.062	27.1875
	Mean ± S. E.	**57.7734^e**	**45.1172^d**	**39.6094^b**	**43.125^c**	**28.418^a**
	(C. V. %)	**± 0.5197**	**± 0.5404**	**± 0.3968**	**± 0.5135**	**± 0.9086**
		(3.5981)	**(4.7913)**	**(4.0074)**	**(4.7628)**	**(12.7893)**
2	Alkaline phosphatase	114.5833	110.0000	100.8333	105.4167	64.1667
		142.0833	100.8333	82.5000	123.7500	59.5833
		132.9167	105.4167	100.8333	142.0833	64.1667
		128.3333	110.0000	110.0000	128.3333	59.5833
		151.2500	100.8333	100.8333	110.0000	50.4167
		105.2500	96.2500	96.2500	123.7500	55.0000
		114.5833	100.8333	100.8333	105.4167	64.1667
		128.3333	114.5833	114.5833	114.5833	50.4167
		146.6667	100.8333	100.8333	119.1667	45.8333
		128.3333	96.2500	91.6667	123.7500	50.4167
	Mean ± S. E.	**129.25^d**	**104.9583^b**	**99.9166^b**	**119.625^c**	**56.3750^a**
	(C. V. %)	**± 4.6741**	**± 1.9861**	**± 2.8005**	**± 3.5797**	**± 2.1660**
		(11.4358)	**(5.9839)**	**(8.8632)**	**(9.4629)**	**(12.1499)**

i. Comparisions were made between the groups
ii. Values with common superscripts do not differ significantly ($p \leq 0.05$)

Table-2: Least Squares Analysis of Variance Showing Effect of Freezing Rates on Leakage of Seminal Plasma Phosphatases in Semen

S. N.	Seminal Plasma Phosphatase	Source of variance	Degree of freedom	MSS Values
1.	Acid Phosphatase	Between groups	4	1786.8054**
		Remainder	75	5.7883
2	Alkaline Phosphatase	Between groups	4	7869.8281**
		Remainder	75	102.2804

Note: Superscript**denotes Significant Differences at 1% level ($P \leq 0.01$)

Frozen Thawed Semen

Acid Phosphatase

Acid phosphatase in seminal plasma of semen from M_1; M_2; M_3; M_4 and control groups were in the range from 54.375 to 60.9375; 41.25 to 48.75; 37.5 to 42.1875; 39.375 to 46.875 and 22.5 to 35.625 KA units per 0.9221 x 10^9 spermatozoa respectively. The Mean $\pm$ S. E. (CV%) values of ACP in respective groups were 57.7734 $\pm$ 0.5197 (3.5981); 45.1172 $\pm$ 0.5404 (4.7913); 39.6094 $\pm$ 0.3968 (4.0074); 43.125 $\pm$ 0.5135 (4.7628) and 28.418 $\pm$ 0.9086 (12.7893) KA unit per 0.9221 x 10^9 spermatozoa respectively (Table-1).

Values of acid phosphatase in seminal plasma of fresh diluted semen (control group) were lower than those from frozen thawed semen from freezing mode groups, the differences were highly significant ($P \leq 0.01$). The percent rise in ACP contents from dilution to frozen thaw stage in M_1; M_2; M_3 and M_4 groups were 129.27; 86.18; 77.24 and 112.20 percent respectively. It indicates that freezing inflicts cryo-injury to spermatozoa with resultant extracellular leakage of phosphatases. Similar findings were reported by Glogowski andStrezezek (1979) and Szasz et al. (2000).

Among freezing mode groups lowest value of seminal plasma ACP was observed in M_3 followed by M_4, M_2 and M_1 groups in increasing order. Significant ($p \leq 0.05$) differences were observed among all freezing mode groups. It reveals significant effects of freezing rates on leakage of ACP. It is in agreement with the opinions of Graham and Pace (1967), Mohan (1982) and Szasz et al. (2000) who stated that freezing rates significantly influence the release of enzymes from spermatozoa.

Alkaline Phosphatase

Alkaline phosphatase contents in seminal plasma of semen from M_1; M_2; M_3; M_4 and control groups were in the range from 105.42 to 151.25; 96.25 to 114.5833; 91.6667 to 114.5833; 105.4167 to 142.0833 and 45.8333 to 64.1667 KA units per 0.9221 x 10^9 spermatozoa respectively. The Mean $\pm$ S. E. (CV %) values in respective groups were 129.25 $\pm$ 4.6741 (11.4358); 104.9583 $\pm$ 1.9861 (5.9839); 99.9166 $\pm$ 2.8005 (8.8632); 119.625 $\pm$ 3.5797 (9.4629) and 56.3750 $\pm$ 2.1660 (12.1499) KA units per 0.9221 x 10^9 spermatozoa (Table-1).

Alkaline phosphatase contents in seminal plasma of fresh diluted semen from control group were lower than those observed in frozen thawed semen from freezing mode groups, the differences were highly significant ($P \leq 0.01$).These results are in accordance with previous reports stating that process of cryo preservation inflicts considerable damage to spermatozoa with resultant higher level of alkaline phosphatase in seminal plasma of frozen thawed semen (Singh *et al.,* 1996 and Szasz *et al.,* 2000).

The percent rise in AKP contents observed from dilution to frozen thaw stage in M_1; M_2; M_3 and M_4 groups were 103.3; 58.76; 39.38 and 51.75 percent respectively. In present study the percent rises in AKP contents from dilution to frozen thawed stage were lower than that reported by Singh *et al.* (1996).

Among freezing mode groups lowest value of AKP in seminal plasma was observed in M_3 followed by M_2, M_4 and M_1 groups in increasing order. Significant ($P \leq 0.05$) differences were observed between M_1 and M_2; M_1 and M_3; M_1 and M_4; M_2 and M_4; M_3 and M_4, whereas, non-significant differences were observed between M_2 and M_3 groups (Table-1). It revealed that freezing rates significantly affects the leakage of AKP. Similar conclusions were made by Graham and Pace (1967) and Mohan (1982).

The mean values of AKP contents in seminal plasma of frozen thawed semen from M_1, M_2, M_3 and M_4 groups were higher than those reported by Singh *et al.* (1996). These variations could be attributed to differences in breed (Sinha *et al., 1985*; Sinha *et al.,* 1988 and Sinha *et al.,* 1999-2000), age of bucks (Tiwari, 2000), seasons of semen collection (Baruah *et al.,* 1992 and Kale and Tomer, 2000), individual variations between

the bucks (Tuli *et al.,* 1991 and Kale and Tomer, 2000), season of semen collection (Baruah *et al.,* 1992), sperm concentration (Kakar and Anand, 1984), rates of dilution and composition of diluents (Singh *et al.,* 1993 and Singh *et al.,* 1996) and freezing rates (Graham and Pace, 1967; Mohan, 1982 and Szasz *et al.,* 2000).

The enzyme release from spermatozoa has generally been viewed as cellular injury (Ingale *et al., 2000*), whereby membrane become inactive with altered permeability or destroyed resulting into loss of material therein (De-Reuck and Knight, 1963 and Guraya 1987). The process of cryopreservation causes diminished intracellular enzyme activity that results from leakage of enzyme into the extracellular surrounding medium. Species release differences (Roychoudhury *et al.,* 1974) have been attributed to intrinsic differences in the cells between the species (White and Well, 1960) as well as differences in susceptibility to membrane damage between the species (Hammerstedt *et al.,* 1978).

Increased value of phosphatases in seminal plasma are attributed to sperm injury inflicted upon by dilution, equilibration and freezing of semen (Glogowski and Strzezek, 1979 and Szasz *et al.,* 2000) as observed in present study.

In-vivo fertility rates with semen from M_1, M_2, M_3, M_4 and control groups were 10.00, 27.27, 36.36, 20.00 and 45.45 percent respectively. It was higher in doe inseminated with diluted semen as compared with those inseminated with frozen thawed semen.

Among freezing mode groups highest fertility rates were observed with semen from M3 followed by M2, M4 and M1 group in decreasing order.

Conclusion

It was concluded that freezing inflicts cryo-injury with resultant increased leakage of phosphatase enzyme into the seminal plasma that adversely affect the fertilizing ability of semen. On the basis of phosphatase leakage and fertility rates optimum freezing rate for cryopreservation of buck semen was observed as 25^0 C/minute.

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